

Analytical Studies on the Higher Fatty Acids of Tobacco*

Crude approximations of the higher fatty acid content of cured Type 12 tobacco have been reported from this laboratory (1). These results indicated that the level of such acids extractable by *n*-hexane and insoluble in aqueous methanol is about 0.65%. Gas chromatography of the methyl esters prepared from these acids showed that the major part of the fraction volatile under the gas chromatographic conditions consisted of methyl palmitate, stearate, oleate, linoleate, and linolenate. This study was one of a series on the composition of tobacco, including quantitative analyses of various types of tobacco for paraffinic hydrocarbons and sterols (2-5) and approximations of the total composition of the hexane-soluble material of flue-cured leaves (6).

In a continuation of these studies, various types and grades of cured tobacco leaves have now been analyzed for the common fatty acids from C_{14} to C_{26} by an improved procedure involving more rapid and convenient methods for the separation of acidic from non-acidic material and for the preparation of the methyl esters for analysis by gas chromatography.

Experimental

Gas Chromatographic Equipment and Procedure.—Aerograph¹ Models A-100 and A-350 were used, and samples were injected with Hamilton microsyringes. All columns were stainless steel spirals 5' long and either $\frac{1}{4}$ " or $\frac{1}{2}$ " o.d. Two column packings were used: 20% (w/w) diethylene glycol succinate polyester coated on 40-60 mesh Chromosorb W in the $\frac{1}{2}$ " column, and 20% (w/w) butanediol succinate polyester on 60-80 mesh firebrick in dual $\frac{1}{4}$ " columns for the Model A-350 in-

strument. Helium flow rates were measured with a calibrated rotameter in the A-100 and with a soap bubble flowmeter in the A-350.

Conditions were similar in both instruments: 200° oven temperature, 60-90 ml of helium per minute, and thermal conductivity cells as detectors. The detector was included in the column oven of the A-100 but was housed in a separately controlled oven at 248° in the A-350. Filament current was set at 250 milliamps in the A-100 and 200 milliamps in the A-350. Injector blocks were controlled at 260° in both instruments. Recorders were standard models operated at 4 or 5 mv full scale with a chart speed of 40" per hour.

Peak areas were measured by triangulation (7). Peak widths at the base were in the range 30-100 mm. Known quantities of the authentic methyl esters were chromatographed daily under the same conditions as the unknowns in order to obtain peak area-concentration relationships and retention time data. The individual peaks were identified by comparative retention times supplemented by infrared spectral examination of collected fractions.

Processing of Tobacco.—Separation of acidic from non-acidic material and *in situ* esterification of the adsorbed acids on alumina were accomplished by a procedure based on that developed by Sammons and Wiggs (8) for the quantitative separation of fatty acids in food, feces, or serum. Cured, aged, or fermented tobacco leaves with midribs removed² were ground in a Wiley mill to pass a 50-mesh screen, and 12.50 g of the air-dried samples were extracted for 21 hours in a Soxhlet apparatus with 250 ml of Skellysolve B (essentially *n*-hexane). The extract was concentrated to approximately 10 ml on a steam bath and mixed with 12.5 g of chromatographic grade alumina. After 15 minutes the alumina was freed of non-acidic material by repeated washes, first with petroleum ether (30-60°) and then with ethyl ether. The alumina was allowed to air-dry, and the adsorbed acids were esterified by covering them with 20 ml of 4% (v/v) H_2SO_4 in methanol and immersing the stoppered flask in a 40° bath overnight.

² Midribs were not removed from the Turkish leaves.

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¹ Mention of a specific company or product does not constitute endorsement by the Department over other companies or products not mentioned.

The alumina was separated by filtration and washed thoroughly on the filter with petroleum ether. The filtrate was transferred to a separatory funnel and the lower layer was extracted repeatedly with petroleum ether. The combined petroleum ether extracts were evaporated almost to dryness, transferred to a tared 15 ml conical centrifuge tube, and weighed. Aliquots of approximately 60 microliters of this solution were weighed in a 100 microliter syringe and injected into the gas chromatograph. To obtain the total weight of methyl esters derived from the original 12.50 g of tobacco, a weighed aliquot was dried to constant weight under a stream of nitrogen at 40° and the residue was weighed.

Results and Discussion

Acids in Type 12 Tobacco.—The above procedure was adopted for use in comparative studies of the free higher fatty acid content of different tobaccos. Acids present as salts remain unextracted under the conditions described. Combined acids (esters, amides, salts of organic bases) are extracted to an extent dependent upon their solubility in hexane but are eluted from the alumina prior to the esterification step. The common nonvolatile tobacco acids may be extracted in varying amounts by hexane, but their methyl esters are more volatile than those of the higher fatty acids and require much lower column temperatures for resolution (9).

Results of experiments in which various other solvents were used for the extraction showed that the amounts of total solids extracted varied widely, but the amounts of acids adsorbed on the alumina were about the same with ether, hexane (Skellysolve B), and ethanol (2.116, 2.146, and 2.228 g of methyl esters per 100 grams of tobacco, respectively). Acetone extracted somewhat less acidic material (1.98 grams). As expected, ether to which HCl had been added extracted more (2.67 grams) than ether alone, since acids combined as salts are included. In one experiment the sum of myristic, palmitic, stearic, oleic, linoleic, and linolenic acids determined by gas chromatography of the methyl esters was about 90% as large with hexane as with ethyl ether.

Suitable modifications of the extraction

and gas chromatographic procedures would make the method applicable to the determination of any desired fraction of the total acids of tobacco, volatile or nonvolatile, combined as well as free. We have shown, for example, that saponification of an aliquot before adsorption on alumina permits determination of free, combined, and total acids, provided the amount of alumina used is adjusted to keep the ratio at about 1 gram per 25 mg of total (free + saponified) acids contained in the aliquot (Table 1). Furthermore, since these data were obtained from an extract prepared with a solvent containing HCl (10), the values for "free" and "total" acids in Table 1 include those derived from salts.

Table 1. Content^a of free and combined acids in Type 12 tobacco

Fraction	g/100 g Tobacco
Total extract (Et ₂ O-HCl)	11.2
Petroleum ether solubles	6.2
Total acids	3.4 ^b
Free acids	2.1 ^b
Combined acids	1.3 ^c
Total palmitic acid	0.14 ^d

^a Single determination on an air-dried basis.

^b Assuming an average molecular weight of 250 for the methyl esters.

^c By difference.

^d By gas chromatography of methyl esters.

The method provides for the removal of less strongly adsorbed material from the alumina before the esterification. This includes volatile neutral compounds such as esters, alcohols, and amides. Interference from this source during gas chromatography is therefore eliminated.

Illustrative data for replicate determinations of the major free higher fatty acids of Type 12 tobacco, using the method described under *Experimental*, are given in Table 2. Recovery of 400 mg of palmitic acid added to a similar sample was 348 mg, or 87%.

Less than half of the preparation of methylated tobacco acids injected into the gas chromatograph was volatile, as shown by collection at the exit. Of this volatile portion about 68% is accounted for as myristic, palmitic, stearic, oleic, linoleic, and

Table 2. Reproducibility in free fatty acid determination^a

Fatty Acid	mg/100 g Type 12 Tobacco ^b			
	Sample I	Sample II	Sample III	Sample IV
Myristic	3	3	3	3
Palmitic	114	82	104	107
Stearic	23	22	23	24
Oleic	29	20	24	22
Linoleic	56	37	46	51
Linolenic	181	143	144	155

^a On hexane extracts (see *Experimental*).

^b On an air-dried basis.

linolenic acids in Table 2. The remaining 32% of the volatile portion appears on the gas chromatograms as a large number of minor components and nondescript background substances that are not separated into sharp peaks under the conditions used. Other types of tobacco gave chromatograms of sufficiently different and characteristic appearance as to suggest their possible use in establishing the identity of unknown samples by a "fingerprint" technique. No attempt has been made to establish positively the identity of these minor components appearing on the chromatograms or of the components of the large portion of the methyl ester preparation from Type 12 tobacco found to be nonvolatile under the gas chromatographic conditions.

Relation of Quality to Higher Fatty Acid Content of Unaged Flue-cured Tobacco.—Differences in grade or smoking quality are obviously reflections of variations in chemical and physical composition of the leaf (11). In this connection it was of interest to determine whether a relationship exists between the level of free higher fatty acids and the quality of tobacco leaves.

Commercial samples were obtained of three types of flue-cured unaged tobaccos, the qualities of which had been judged as low, medium, and high grade. Samples from two successive crop years were also included. Table 3 presents representative results. The moisture content of all samples was approximately 10% and correction to a moisture-free basis would not significantly alter the indicated pattern. In general, the low-quality samples tend toward a

lower fatty acid content, but differences are slight and inconsistent, except perhaps in the case of linolenic acid. Differences between medium and high grades are not significant except in the Type 13, 1958 crop. Variations between growing areas and between the two crop years are at least as great as between grades.

Acids of Aged or Fermented Tobaccos.—Reports on the fatty acids of green and flue-cured tobacco and smoke have been reviewed (12). One such investigation showed the presence in flue-cured cigarette tobacco of all the saturated straight-chain acids from C₁₀ to C₂₃ (except C₁₃) and some unsaturated acids; the predominant acids were palmitic, stearic, linoleic, and linolenic, with smaller quantities of oleic, arachidonic, and other unsaturated acids (13). Table 4 compares aged flue-cured ("Bright") tobacco with a number of other types with respect to the content of the major higher fatty acids.

The fatty acid mixtures in all types were found to be complex, judging from the number of peaks on the chromatograms. No attempt at positive identification of minor components was made, but the fractions volatile under the gas chromatographic conditions consisted of 10 to 14 components with retention times below that of C₁₄, two to four between C₁₄ and C₁₆, two to six between C₁₆ and C₁₈, and one or more beyond linolenate, for a total of 15–25 minor components not listed in Table 4. The total numbers of acidic compounds in the volatile fractions are even larger, since the gas chromatographic conditions selected did not permit differentiation of esters below about C₁₀ or above C₂₀. Comparison with known retention times indicates that not only are straight-chain saturated acids present among the unidentified minor components in the C₁₀ to C₂₀ range but probably branched and unsaturated acids also, such as those several workers have reported to be present in cigarette smoke condensates (14, 15).

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Table 3. Content^a of free higher fatty acids in unaged flue-cured tobacco

Fatty Acid	mg/100 g Tobacco					
	1957 Crop			1958 Crop		
	Grade			Grade		
	Low	Medium	High	Low	Medium	High
Type 11a, Old Belt						
Myristic	1.9	2.4	1.9	2.1	1.4	1.7
Palmitic	68	94	90	104	98	103
Stearic	8	11	10	39	31	31
Oleic	7	17	10	14	13	16
Linoleic	49	56	50	60	56	52
Linolenic	85	114	110	126	133	157
Type 12, Eastern North Carolina Belt						
Myristic	2.4	2.0	2.0	2.9	2.2	1.8
Palmitic	98	103	125	112	124	113
Stearic	29	26	33	16	16	16
Oleic	15	15	17	13	16	16
Linoleic	48	51	52	65	59	51
Linolenic	122	126	159	134	157	152
Type 13, South Carolina Belt						
Myristic	2.6	—	2.4	2.6	1.6	2.7
Palmitic	109	—	135	132	126	153
Stearic	37	—	35	41	36	41
Oleic	13	—	17	28	14	18
Linoleic	48	—	56	41	53	62
Linolenic	133	—	185	180	182	227

^a On an air-dried basis.

Table 4. Content^a of certain higher free fatty acids in aged or fermented tobacco

Fatty Acid	mg/100 g Tobacco						
	Bright	Burley	Maryland	Turkish	Fire-Cured	Cigar Filler	Cigar Binder
Myristic	3	2	3	5	2	3	4
Palmitic	101	51	44	103	32	41	59
Stearic	18	8	12	12	18	8	18
Oleic	15	5	11	17	3	5	7
Linoleic	53	25	35	51	17	8	20
Linolenic	110	35	21	78	20	2	21

^a Single determinations on an air-dried basis.

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